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(54) **PEPTIDE WITH ACTIVITY OF INHIBITING PHOSPHOLIPASE A 2? ORIGINATING IN INFLAMED PART.**

(57) A peptide with an activity of inhibiting phospholipase A_2 originating in inflamed parts, which has an amino acid sequence represented by formula (I).

Gln Lys Asp Ala Pro Asp His Gln Glu Leu Asn Leu
1 5 10

Asp Val Ser Leu Gln Leu Pro Ser Arg ... [I]
15 20

EP 0 506 971 A1

Technical Field

The present invention relates to peptide inhibitors of phospholipase A_2 purified from inflammatory sites.

Background Art:

Phospholipase A_2 is an enzyme which hydrolyzes β-ester bonds in phospholipid to give fatty acids and lysophospholipids. Especially, it releases arachidonic acid which can be a precursor of prostaglandins, leukotriene, thromboxane and the like from membrane phospholipids and is thought to play an important role in the production of these inflammatory mediators. In recent years, phospholipase A_2 has been purified from the inflammatory sites of human inflammatory diseases and inflammatory model animals (it will be called phospholipase A_2 from inflammatory sites) and its properties have been clarified. This enzyme is thought to have the action to accelerate the inflammatory reactions, therefore the drug to prohibit the activity of this enzyme can be expected to reveal anti-inflammatory actions.

Complement C3 is a protein which has been known to perform the key functions in the complement pathway. C3 is hydrolyzed stepwise by a protease in blood. In other words, it is cleaved first with convertase into C3a and C3b. C3b binds through its thiol ester site to the surface of activators followed by activation of the complement pathway to form a membrane attack complex. C3a works as anaphylatoxin. At this time, a minor part of C3b binds to the activators, while the major part reacts with water to lose the binding activity, further undergoes hydrolysis by a protease to convert into C3dg or C3d finally.

The present inventors have already applied patents after finding that human and rat C3dg inhibit specifically phospholipase A_2 purified from inflammation sites, succeeding in expression of rat C3 cDNA in Escherichia coli to produce a part of rat C3α chain (containing the C3dg part) as a recombinant protein, and realizing that the recombinant protein inhibit specifically phospholipase A_2 purified from inflammatory sites (PCT/JP90/00996, W091/01999).

Human C3dg is, however, a protein of about 39 kDa molecular weight and it can be anticipated that the use of said protein as an anti-inflammatory as such will cause troubles in, for example, the transference to the affected part, the storage stability or antigenecity.

Thus, a novel peptide having inhibitory activity against phospholipase A_2 from inflammatory sites is desirably provided as an anti-inflammatory without such troubles.

Hereupon, the present inventors have made intensified studies in order to solve the above-mentioned problems to find that a part of the amino acid sequence of C3dg have action to inhibit phospholipase A_2, and attained the present invention.

Disclosure of the Invention:

In other words, the present invention is a peptide inhibitor of phospholipase A_2 purified from inflammatory sites having an amino acid sequence shown in sequence No. 1.

Brief Description of the Drawings:

Fig. 1 gives the fractionation of the peptide in Example 1 according to the present invention by means of reversed phase HPLC.

Fig. 2 gives the inhibitory activity of the peptide according to the present invention against phospholipase A_2, which was determined in Example 2.

In these figures, ●-● show the cases where the phospholipase A_2 purified from human inflammatory sites, O-O give the phospholipase A_2 from rat inflammatory sites and x-x show the phospholipase A_2 from porcine pancreas.

Best Embodiment of the Invention:

The peptides include the amino acid sequence of 21 residues shown in the sequence No. 1. The amino acid sequence of said peptide is identical with the amino acid sequence from No. 612 to the C-terminus of human C3α chain. In the amino acid sequence in sequenc No. 1, the peptides having substitution, deletion or insertion of one or more amino acid residues are also included in the peptides according to the present invention as long as they have the inhibitory activity against phospholipase A_2 from inflammatory sites.

Such peptides according to the present invention can be obtained by synthesis according to a customary procedure described in, for example, "The basis and experiments in peptide syntheses" (written

in Japanese); N. Izumiya, T. Kato, H. Aoyagi and M. Yaki: Maruzen, Tokyo) or E. Atherton, R.C. Sheppard; "Solid phase peptide synthesis, a practical approach" (LRL press) followed by purification. Or human C3α chain or the like is used as a starting substance to be hydrolyzed with an enzyme such as α-chymotrypsin.

The peptide synthesis, the measurement of the inhibitory activity against phospholipase A_2 or the like used in the present invention will be illustrated in the following:

① Peptide synthesis and purification

A peptide synthesizer 431A of Applied Biosystem was employed to conduct the synthesis by the Fmoc method.

The deprotection of the samples was carried out by the TMSBr cleavage method according to the protocol of Applied Biosystem.

The samples were purified by using the reversed phase HPLC column (Vydac Protein C_{18}, 2.2 cm ID x 25 cm L) and eluted with the gradient of 0 to 80 % acetonitrile in the presence of 0.1 % trifluoroacetic acid.

② Determination of inhibitory activity against phospholipase A_2

The activity-measuring system was prepared by adding distilled water to 100 mM Tris-HCl (pH 9.0), 4 mM calcium chloride, 0.1 mM [^{14}C] phosphatidylethanolamine (2,000 dpm/nmol), and 10 μl sample to adjust the total volume to 240 μl, and finally 10 μl of 0.1 ng/μl phospholipase A_2 was added. The phospholipases A_2 used were from human inflammatory sites (the joint fluid from patients with rheumatoid arthritis) (Hara et al., J. Biochem., 104, 326-328, 1988), rat inflammatory sites (Chang et al., J. Biochem., 102, 147-154, 1987), and porcine pancreas (Boehringer Manheim Co.). Phosphatidylethanolamine was purified from Escherichia coli cultured in a medium to which [^{14}C] acetic acid was added.

The reactions were conducted at 37°C with stirring for 10 minutes. The termination of the reaction and the extraction of fatty acids generated were carried out by the Dole's method (Dole et al., J. Biol. Chem., 235, 2595 - 2599, 1960) and the extracted [^{14}C] fatty acid was determined with a scintillation counter.

The present invention will be illustrated in more detail by Examples.

Example 1 (Synthesis and purification of the peptides having inhibitory activity against phospholipase A_2 from inflammatory sites)

A peptide was synthesized in accordance with the amino acid sequence given in sequence No. 1 using a peptide synthesizer (Applied Biosystem, 413 A). The yield was 77.9%.

After deprotection, the 140.7 mg crude product was fractionated with reversed phase HPLC (Fig. 1). The main peak eluted with about 35 % acetonitrile was collected and lyophilyzed. The yield of the finally purified sample was 21.8 mg. The sample was confirmed to have the amino acid sequence of No. 1 by a gas-phase protein sequencer 477 A of Applied Biosystem.

Example 2 (Phospholipase A_2 inhibitory activity)

The lyophilyzed sample obtained in Example 1 was dissolved again in 50 % acetone-0.1 % trifluoroacetic acid to adjust the concentration to 10 mg/ml. The solution was further diluted stepwise with the same buffer and the inhibitory activity of phospholipase A_2 from inflammatory sites was determined at individual concentrations.

The results are given in Fig. 2. The peptide according to the present invention inhibited phospholipase A_2 from human inflammatory sties (●-●) and phospholipase A_2 from rat inflammatory sites (O-O) dose-dependently. In both enzymes, the amount of the protein needed for 50 % inhibition of 1 ng enzyme activity was about 300 ng and IC_{50} was about 5×10^{-7} M. Meanwhile, it showed no inhibitory activity against phospholipase A_2 from porcine pancreas (x-x).

Field of Industrial Utility

Peptide inhibitors of phospholipase A_2 from inflammatory sites according to the present invention have inhibitory activity against phospholipase A_2 from the inflammatory sites and are expected to have an action to inhibit the allergic reactions, can be utilized as an anti-inflammatory and a therapeutic agent for allergic diseases in mammalians especially in human. Additionally, they are expected to have excellent transference to the affected parts and high storage stability with reduced troubles.

[Table of amino acid sequence]

Sequence Number: 1

Sequence Length: 21

Type of sequence: Amino acid sequence

Topology: Straight-chained molecule

Kind of sequence: Protein

Type of fragment: Intermediate fragment

Sequence:

Gln	Lys	Asp	Ala	Pro	Asp	His	Gln	Glu	Leu	Asn	Leu	Asp	Val
1				5					10				

Ser	Leu	Gln	Leu	Pro	Ser	Arg
15				20		

Claims

1. Peptide inhibitors of phospholipase A_2 purified from inflammatory sites comprising the amino acid sequence given in sequence No. 1.

2. The peptides according to claim 1 wherein said amino acid sequence includes one or more substitutions, deletions and insertions of amino acid residues.

FIG. 1

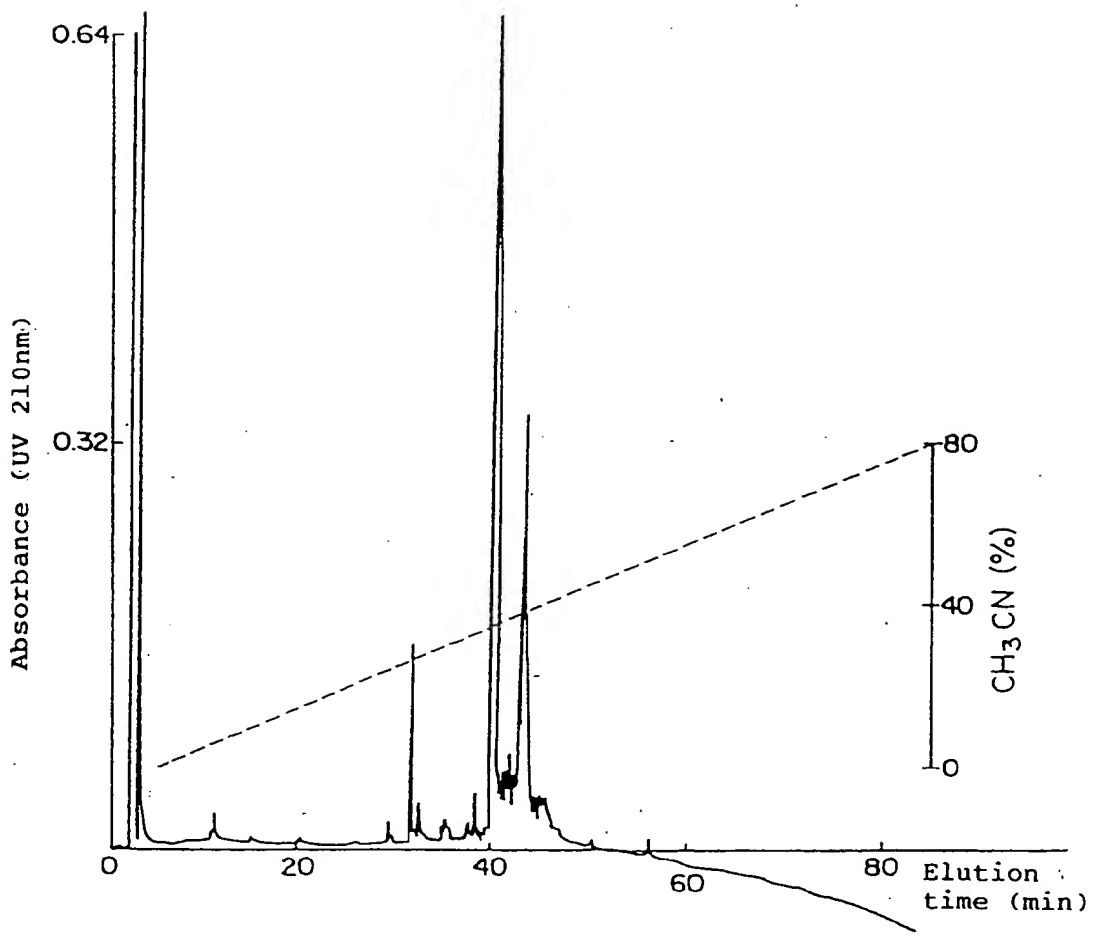
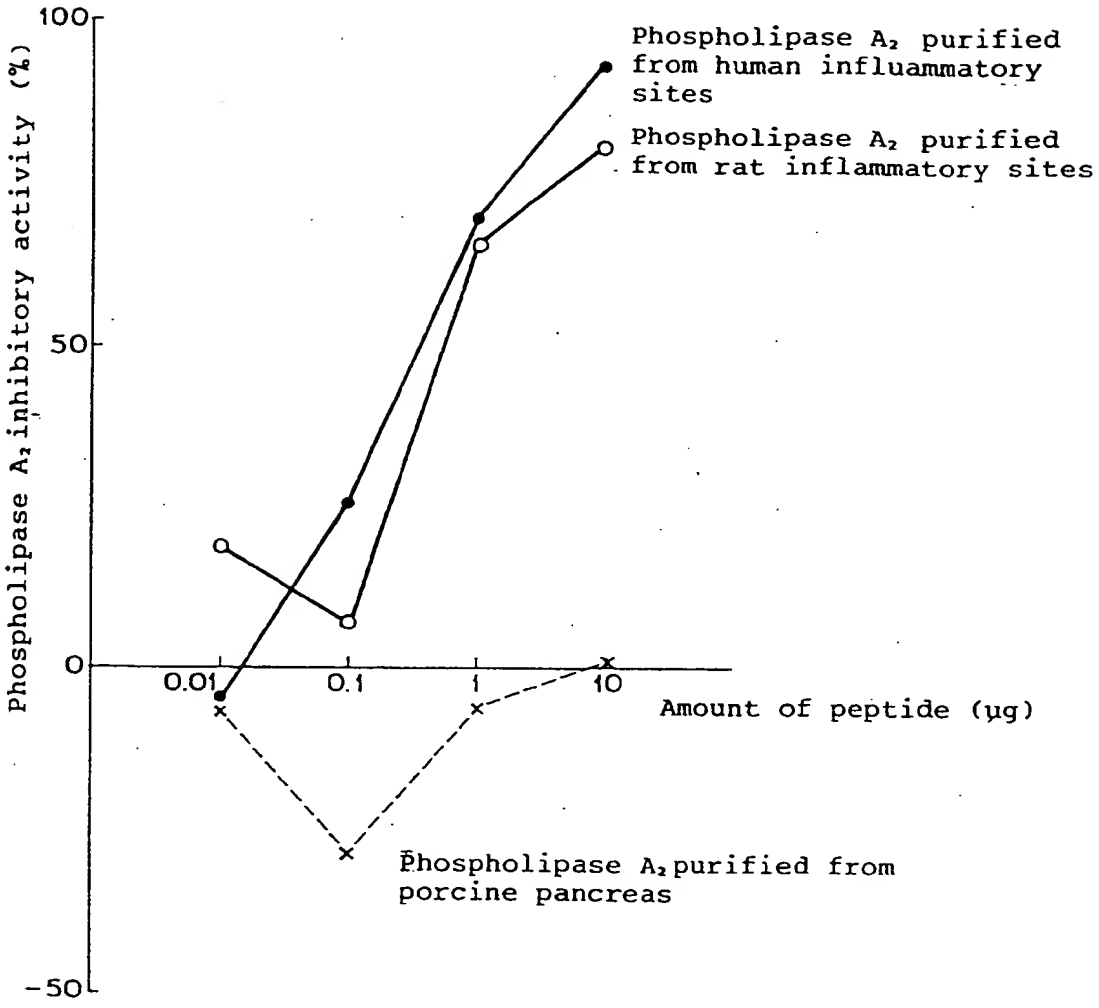

0.64
0.32
Absorbance (UV 210nm)
0
20
40
60
80
Elution time (min)
80
40
0
CH_3CN (%)

FIG. 2


100
50
0
-50
0.01
0.1
1
10
Phospholipase A₂ inhibitory activity (%)
Amount of peptide (μg)
Phospholipase A₂ purified from human influammatory sites
Phospholipase A₂ purified from rat inflammatory sites
Phospholipase A₂ purified from porcine pancreas

INTERNATIONAL SEARCH REPORT

International Application No PCT/JP91/01424

I. CLASSIFICATI N OF SUBJECT MATTER (If several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl[5] C07K7/10, A61K37/64, C12N9/99

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C07K7/00, A61K37/00, C12N9/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Biochem. J., Vol. 230, No. 2 (1985) Ulf Hellman et al. "Amino acid sequence of the trypsin-generated C3d fragment from human complement factor C3" pp. 353-361	1-2
A	Proc. Natl. Acad. Sci. U.S.A., Vol. 82, No. 3 (1985) Maarten H. L. De Bruijn et al. "Human complement component C3 : cDNA coding sequence and derived primary structure" pp. 708-712	1-2

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
December 12, 1991 (12. 12. 91)	January 14, 1992 (14. 01. 92)
International Searching Authority	**Signature of Authorized Officer**
Japanese Patent Office	